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BRIEF COMMUNICATIONS

Evaluation of a non-invasive tagging system for laboratory studies using three-spined sticklebacks *Gasterosteus aculeatus*

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A non-invasive tagging system for individual identification of three-spined sticklebacks *Gasterosteus aculeatus* was evaluated. The tags were easily detected *via* video, and tagged and non-tagged fish did not differ in terms of growth, activity levels or shoaling behaviour.

Key words: individual identification; methodology; social behaviour; welfare.

Animal welfare legislation favours the reduction and replacement of invasive procedures. Invasive tagging procedures, in which tags that are attached to animals penetrate or damage the epidermis and body cavity, can potentially lead to stress and mortality through injury or infection of the tagging site (FSBI, 2002). Numerous tagging methods for fishes have been developed, to meet the various demands of researchers interested in aspects of fish ecology such as migration, dispersal, ranging patterns and site fidelity, and behaviours including shoaling preferences and other social interactions. Surgically implanted telemetry and passive integrated-transponder (PIT) tags are widely used to remotely study free-ranging fishes. Such tags provide a reliable method of monitoring individuals. Mortality is generally low, and tag retention high, with performance being influenced by factors such as fish size (and more specifically the tag mass to fish mass ratio), fish morphology and environmental conditions (Baras *et al*., 2000; Jepsen *et al.,* 2002). Whilst these tags represent a valuable means of tracking free-ranging fishes, they are used to a much lesser extent in the laboratory, where individuals can usually be tracked visually and where cheaper alternative tagging methods are often available. Implanted microtags, such as injected coloured elastomers and coded wire tags, and subcutaneous dyes represent such alternatives. Both are widely used in the laboratory as well as in the field, and numerous studies have reported low mortality and high retention rates among tagged

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fishes, even in very small individuals (Beukers *et al.,* 1995). A potential disadvantage of using microtags and dyes is that it may not always be possible to detect the marks at range, meaning that individuals may have to be inspected closely or even captured before they can be identified. While this may not be a problem under most circumstances, it is a concern when conducting behavioural experiments that call for fishes to be observed at length without being disturbed. Finally, another method for identifying individuals is to use ectoparasites as natural tags (MacKenzie, 2002). While this approach has been used widely in population studies, it may be unsuitable for use in certain behavioural studies, since many parasites are known to affect aspects of fish behaviour (Barber & Rushbrook, 2008).

In this study, a non-invasive tagging method for use with the three-spined stickleback *Gasterosteus aculeatus* L. was evaluated. *Gasterosteus aculeatus* is a widely used and well-established model organism in behavioural ecology (Bell & Foster, 1994), owing in part to its widespread distribution through the temperate northern hemisphere, which encompasses a wide range of habitat types and corresponding adaptive diversity, and the relative ease with which it can be collected, housed and manipulated in the laboratory. *Gasterosteus aculeatus* have proven to be especially useful in the study of many aspects of social behaviour. Often, such studies call for experimenters to be able to quickly recognize different individuals or classes of individuals (Whitehead, 2008), necessitating the use of some form of tag as an identifier. The tagging system tested here comprises disc-shaped tags mounted upon the dorsal spines of the fish. The tags can easily be modified to carry letters or symbols that can be read *via* high-resolution video cameras under normal lighting conditions, allowing for rapid identification. While similar tagging procedures (Barber & Ruxton, 2000; Ward *et al.,* 2002) and even spine-attached leashes (Östlund-Nilsson & Nilsson, 2000) have been used with this species previously, to date there has been no rigorous study of the effects of spine-mounted tags on the welfare or behaviour of the fish. In this study, the effects of spine-mounted tags on the growth, activity levels and shoaling behaviour of *G. aculeatus* were investigated, comparing tagged and non-tagged fish.

Fish measuring 45 ± 2 mm (mean $\pm$ S.E.) standard length (L_S) were captured using mesh cage traps from the Kinnessburn Stream, St Andrews, U.K. (56° 20′ 05″ N; 2° 47′ 14″ W) in September 2008. The fish were held in the laboratory for 1 month at 8° C in groups of 20 before being tested in 45 l aquaria. They were fed once per day with frozen chironomid *Chironomus* sp. larvae (Ocean Nutrition; www.oceannutrition.com). Following the completion of the experiments outlined below, the fish were retained in the laboratory for use in a separate study.

Circular tags measuring 5 mm in diameter and weighing *c*. 10 mg were cut from sheets of PVC insulation tape (brand: 3M Temflex™; www.3m.com). The tape was double layered, with the adhesive surfaces pressed together. Colours in the red spectrum, the male nuptial colour in *G. aculeatus*, were avoided since both sexes are intrinsically attracted to red objects (Smith *et al*., 2004). White, brown and green tags were used. Tags were pierced with a 0·4 mm needle and placed over the first dorsal spine of the fish. The posterior-facing edge of the first dorsal spine is serrated, and these serrations were sufficient to hold the tag in place without the aid of an adhesive. A membrane at the base of the posterior-facing edge of the first dorsal spine extends up to *c*. 20% of its height. Care was taken not to damage this membrane, since this could potentially lead to infection. The tags were positioned so that they

sat *c*. 40% from the tip of the spine. The fish was held out of water while the tag was placed over its spine. The tagging procedure took *c*. 20 s per fish. No sedation was used as this can lead to stress and mortality in fish as small as those used in this study. Following this, they were returned, in groups of 10 to 45 l aquaria containing aerated water, where they were allowed to recover for 2 h.

The first experiment focused upon growth. Two groups of 28 fish were established. One group was tagged as described above, whereas the other was not. The fish in these groups did not differ in starting mass (mean ± S.E.: tagged, 0·71 ± 0·05 g; non-tagged, 0·70 ± 0·06 g, one-way ANOVA, $F_{1,52}$, $P > 0{\cdot}05$). Fish from within each group were assigned in pairs to their own 30 l housing tank. Each tank contained a 20 mm deep layer of gravel and an air powered filter. The tanks were screened with black plastic sheeting to minimize outside disturbance. The fish were fed daily with frozen *Chironomus* sp. larvae (Ocean Nutrition). The mass of each fish was recorded again prior to feeding on days 4 and 28, after which the experiment ended, and the mean mass for each pair was calculated. A repeated-measures ANOVA revealed that mean mass did not change over time in either group (day 4: tagged, 0·69 ± 0·05 g; non-tagged, 0·69 ± 0·06 g; day 28: tagged, 0·72 ± 0·08 g; non-tagged, 0·71 ± 0·07 g, $F_{1,28}$, $P > 0{\cdot}05$). There was no evidence of either fungal infection or tissue necrosis in either tagged or non-tagged fish over the study period.

The second experiment investigated the effects of the tags upon fish activity. Fish were tested alone in a 90 l aquarium containing a 20 mm deep layer of gravel. The fish was introduced into the centre of the tank in a 100 mm × 100 mm square transparent holding unit and allowed to settle for 5 min. Following this, the holding unit was raised, releasing the fish. Activity, whether the fish was swimming or stationary on the gravel substratum, was point sampled at 20 s intervals for 10 min. This generated 30 recordings per fish. An activity index, defined as the proportion of sampling instances in which they were active was calculated for each fish. Fifteen tagged and 15 non-tagged fish were tested. Comparison of their activity indices revealed that they did not differ (proportion of trial time active: tagged, 0·60 ± 0·10; non-tagged, 0·63 ± 0·07, one-way ANOVA, $F_{1,28}$, $P > 0{\cdot}05$).

The third and fourth experiments considered the effect of the tags upon shoaling preferences. In the third experiment, tagged and non-tagged focal fish were given a binary choice between shoaling with a group of three tagged (one each of white, brown and green tags) *v*. a group of three non-tagged stimulus fish. Fish were tested in a 90 l aquarium (base 900 mm × 300 mm, water depth 120 mm) containing a 20 mm deep layer of gravel. The tank was divided into thirds of 300 mm × 300 mm each using lines drawn on the front. A stimulus chamber, a 100 mm × 100 mm square, 150 mm tall transparent holding unit was located 100 mm from either end of the tank. These were constructed from perforated, colourless plastic, allowing the exchange of visual and chemical cues between the main tank and the chamber. These contained the stimulus fish. The focal fish was introduced into the centre of the tank in a 100 mm × 100 mm square transparent holding unit and allowed to settle for 5 min. Following this, the holding unit was raised, releasing the fish. Point sampling at 20 s intervals for 10 min was used to record which third of the tank the focal fish was in. For tagged and non-tagged focal fish, the amount of time spent in the third of the tank containing the stimulus shoal minus the amount of time spent in the third of the tank containing the non-tagged stimulus shoal was compared against a null expected value of zero, using Wilcoxon signed rank tests. Neither tagged nor

non-tagged focal fish displayed a preference for either stimulus group (proportion of time shoaling with tagged shoal minus proportion of time shoaling with non-tagged shoal: tagged, $-0{\cdot}07 \pm 0{\cdot}01$; non-tagged, $0{\cdot}08 \pm 0{\cdot}01$, $n = 15$, $P > 0{\cdot}05$ for both). Furthermore, a Mann–Whitney U-test revealed that tagged and non-tagged fish did not differ in the total proportion of time they spent shoaling with either stimulus group (tagged, $-0{\cdot}70 \pm 0{\cdot}09$; non-tagged, $0{\cdot}73 \pm 0{\cdot}04$, $n = 15$, $P > 0{\cdot}05$).

The fourth experiment looked at shoaling preferences when all fish were free to move in the test arena. Groups of 10 fish, five tagged and five non-tagged, were placed in a circular arena (diameter 450 mm, water depth 80 mm). Ten groups were tested. Three groups contained two white, two brown and one green-tagged fish, three contained two white, two green and one brown-tagged fish, and four contained two brown, two green and one white-tagged fish. Following a 10 min settling period, these were filmed from above for 30 min. The tag status of the nearest neighbour of each fish, *i.e.* whether it was tagged or non-tagged, was recorded every 3 min, yielding 10 samples of 10 measurements each per group. A pilot study revealed that 3 min was sufficient time for fish to cross the arena multiple times and to associate with several different group mates, ensuring that nearest neighbour at time T_2 was independent of nearest neighbour at time T_1. The proportion of incidences of two tagged or two non-tagged fish occurring as nearest neighbours at each sampling point was recorded. This generated an assortment index where 1 would describe a group where tagged and non-tagged fish clustered with their own kind and 0 would describe a group where tagged and non-tagged fish were alternately arranged within the shoal. A score of 0·5 would therefore indicate an intermediate arrangement of individuals within the group, as would be expected if assortment were unaffected by the presence of tags on some fish. These data were compared against a set of randomly generated null data. This was created by randomly generating 10 000 binary data points and randomly assigning them into 1000 groups of 10, in order to generate assortment indices. These in turn were randomly arranged into 100 groups of 10 indices each, forming the 10 sampling intervals for comparison against the experimental data. As expected, this yielded null data where the mean association index score for each simulated sampling period was 0·5. The experimental and null data were compared using a repeated measures ANOVA to investigate the association patterns of the tagged and non-tagged fish over the 30 min observation period. It was seen that the association indices of mixed groups of tagged and non-tagged fish did not differ significantly from the null data set ($F_{1,108}$, $P > 0{\cdot}05$), and did not change over time ($F_{1,108}$, $P > 0{\cdot}05$), confirming that the tags had no effect on shoaling partner preferences.

This procedure represents a cheap, easy to manufacture, low-stress, and reversible method for tagging *G. aculeatus* and other species with defensive spines. Tagged fish were easily identified, both directly and from video recordings. Four experiments revealed no adverse effects of tagging on fish behaviour or development, and tagged fish did not differ from non-tagged fish in terms of growth, activity levels or shoaling behaviour. No tagged fish showed any signs of ill health throughout the experimental period. These findings, combined with the fact that the tags can be fitted in <20 s, and without breaking the skin of the fish, imply that the method can legitimately be regarded as no more stressful a husbandry procedure than measuring or weighing. In countries such as the U.K., where licences are required from institutions such as the U.K. Home Office for procedures that impose pain, stress or discomfort to

animals, this non-invasive low-stress procedure could be of considerable benefit to both researcher and fish. This investigation suggests that this method of tagging could be an effective way of recognizing individuals or treatment groups in behavioural studies that call for multiple fishes to be observed together. It is envisaged that this could be a very useful tagging method for researchers working with this widely used model organism. Whilst the anatomy of *G. aculeatus* makes them particularly suited for this kind of tag, there is no reason in principle why this tagging system could not be adapted for use with other species that possess similar spines.

While these tags are suitable for use under most laboratory conditions, potential limitations exist. First, these tags were designed to be detected visually; however, this might not be possible under conditions of high turbidity or low light intensity. Second, it is possible that additional drag imposed by these tags might affect the swimming energetics of fishes under flowing water conditions, which may have implications for mortality, as well as sublethal effects upon growth and behaviour. Third, it is possible that the tags might impede movement through dense vegetation, such as filamentous algae. Fourth, the tags might influence risk of detection by predators, where they are present, by increasing the conspicuousness of tagged fishes. Fifth, there may be implications for fish health if care is not taken to avoid damaging the membrane at the base of the spine and if they are to be housed elevated temperatures.

Further work is required to address these potential limitations. If spine-mounted tags are found to have detrimental effects upon fish health or behaviour under these conditions, then other tagging methods, such as PIT tags or sub-dermal implants should be considered.

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